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Liquid and Gas Chromatographic Multi-residue Pesticide Determination in Animal Tissues

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Abstract: A liquid chromatography multi-residue method with photometric detection has been developed. The method is applicable to the quantitative determination of organochlorine (tetradifon, dicofol, chlorfenson, chlorobenzilate), organophosphorus (fenitrothion, azinphos-ethyl) and carbamate (pirimicarb) pesticides in animal tissues. The extracted residues are cleaned up by gel-permeation chromatography. A further fractionation on silica Sep-Pack cartridges is included in the procedure. A gas chromatographic method with electron-capture detection for the analysis of the same pesticides was carried out and the results in the two cases compared. Lower detection and quantitation limits and similar recoveries of pesticides from spiked pig liver and brain samples were obtained by the LC method.

Key words: liquid chromatography, gas chromatography, multi-residue pesticides, animal tissues

1 INTRODUCTION

Pest control in modern agriculture includes treatment of crops pre- and post-harvest with a variety of chemicals, such as herbicides and insecticides in the pre-harvest stage and with fungicides and rodenticides in the storage stage of the total harvest process. Consequently, the residue analysis of herbicides, insecticides, rodenticides and fungicides for quality control purposes is of great interest.

Determination of pesticide residues is usually carried out by gas chromatography (GC). Liquid chromatography (LC) has also been used for pesticide residue analysis, but most of the methods only deal with one class of pesticide, such as carbamates,[1,2] organochlorine[3–5] or organophosphorus.[6,7] Only in a few cases have several classes of pesticide been determined using one system[8,9] or LC been used in combination with GC for multi-residue analysis.[10] LC offers some unique advantages for residue analysis[11] and its potential for multi-residue analysis has not yet been totally exploited. Trace levels of pesticides, using a multi-residue screening procedure, have been detected by various element-sensitive detectors, leading to reliance on mass spectrometry because of its ability to help to define the structure. This has especially been true in the case of the combination of gas chromatography with mass spectrometry (GC/MS). One of the obvious fields of application for this technique was the analysis of pesticide residues in food of animal and of plant origin.[12,13]

In the present study, an alternative LC method is developed for the multi-residue analysis of pesticides that display UV-visible absorbance, in samples of animal origin. Pesticide residues are usually not only monitored in crops, fruits and vegetables, but also in animal tissues where residues may occur by transmission through the food chain. As part of the extensive safety evaluation of agrochemicals, studies to establish the levels of parent compounds and their metabolites which may be passed *via* the food chain into the edible portions of animal tissues[11,14,15] are carried out by industry.

During the development of the liquid chromatography method described in this current study, the following pesticides were examined: organochlorines [tetradifon (4-chlorophenyl 2,4,5-trichlorophenyl sulfone), dicofol (2,2,2-trichloro-1,1-bis(4-chlorophenyl)

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ethanol), chlorfenson (4-chlorophenyl 4-chlorobenzenesulfonate) and chlorobenzilate (ethyl 4,4′-dichlorobenzilate)], organophosphorus (fenitrothion (*O*,*O*-dimethyl *O*-4-nitro-*m*-tolyl phosphorothioate) and azinphos-ethyl (*S*-(3,4-dihydro-4-ozobenzo[*d*][1,2,3,] triazin-3-ylmethyl) *O*,*O*-diethyl phosphorodithioate)) and carbamates (pirimicarb (2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate)). The results obtained by the LC method are compared with those produced using a multi-residue gas chromatographic method with electron-capture detection.

2 EXPERIMENTAL METHOD

2.1 Materials

2.1.1 Instruments

Liquid chromatograph: Pump, L-6200 (Merck-Hitachi, Darmstadt, Germany); autosampler AS-4000 (Merck-Hitachi); detectors: (a) L-4250 UV-visible (Merck-Hitachi); software D-6000 HPLC Manager (Merck-Hitachi); (b) diode-array detector Model ABI-1000S (Applied Biosystems, Ramsey, NJ); software Labcalc (Galactic, Salem, NH).
LC column: Lichrospher 100 RP-18, 125 × 4 mm ID, 5 μm spherical particle (Merck).

Clean-up columns: (1) Glass column 500 × 15 mm ID, slurry packed with Bio-Beads SX-3 (10 g dry), 200–400 mesh (Bio-Rad Labs., Watford, UK) in hexane + chloroform + acetone (75 + 20 + 5 by volume) to a bed height of 360 mm. The mobile phase was supplied by gravity. The column was calibrated with each pesticide eluted with hexane + chloroform + acetone (75 + 20 + 5 by volume). Fractions of 1 ml and increments to 120–140 ml were collected. The pesticides eluted at 50–100 ml. The first 30 ml of eluant were discarded and the next 80 ml were collected. (2) Sep-Pack silica (Waters Assoc., Hartford, UK) 1 g cartridge.

Gas chromatograph: Konik Model KNK-3000 (Konik Instruments, Barcelona, Spain) equipped with electron-capture detector. Operating conditions: splitless injector 250°C, detector 350°C. Fused silica columns: BP5 (SGE) stationary phase 5% diphenyldimethylsiloxane, 25 m × 0·22 mm ID, 0·25 μm film thickness; maintained at 210°C for 1 min, increased to 250°C at 2°C min^{-1} and kept for 10 min; He carrier gas, 10·5 ml min^{-1}; N_2 makeup, 70 ml min^{-1}.

Kitchen mixer: Osterizer Liquefier-Blender (Wisconsin, USA) with a metal container.

Rotary vacuum evaporator: Buchi with thermostatic water-bath and vacuum pump.

2.1.2 Reagents

Acetone, hexane, chloroform were analytical reagent grade (Merck). Methanol and acetonitrile were Lichrosolv gradient grade (Merck). Water was distilled and deionized or Lichrosolv grade. Anhydrous sodium sulfate (Carlo Erba, Milano, Italy) was treated by heating overnight at 350°C. Pesticides were purchased from Dr Ehrenstorfer, Augsburg, Germany or Riedel-de Haën, Hannover, Germany. The solutions were prepared by dissolving in methanol. Working standard solutions were prepared by dilution in methanol. Safety considerations: appropriate precautions should be used to avoid inhalation of chloroform vapour.

2.2 Methods

2.2.1 Extraction from liver

The weighed tissue (20 g) was chopped, dried by admixture with anhydrous sodium sulfate (5 g) and homogenized with a kitchen mixer for 10 min with chloroform + acetone (1 + 1 by volume; 100 ml). The extract was filtered through a glass fibre filter (No. 3) and washed with chloroform + acetone (1 + 1 by volume; 2 × 10 ml). This extract was evaporated to dryness in a rotary vacuum evaporator at 35°C and redissolved in hexane + chloroform + acetone (75 + 20 + 5 by volume) up to 10 ml.

2.2.2 Extraction from brain

The weighed tissue (20 g) was chopped, dried by admixture with anhydrous sodium sulfate (5 g) and homogenized with a kitchen mixer for 10 min with chloroform + acetone (1 + 1 by volume; 100 ml). Total homogenization was achieved through the use of an ultrasonic probe (Branson Sonifier 250) for 15 min at maximum power, the blend centrifuged at 4000 rev min^{-1} for 1 h and the supernatant transferred to a flask. The contents were evaporated to dryness in a rotary vacuum evaporator at 35°C and redissolved in hexane + chloroform + acetone (75 + 20 + 5 by volume) up to 10 ml.

2.2.3 Clean-up

The extracts (5 ml) were transferred to the gel permeation column and the pesticides eluted with hexane + chloroform + acetone (75 + 20 + 5 by volume). The first 30 ml of eluant were discarded, the next 80 ml collected and concentrated to 1 ml using a rotary vacuum evaporator at 35°C and then transferred to a Sep-Pack silicagel cartridge (the Sep-Pak cartridge pre-washed with hexane + chloroform + acetone (75 + 20 + 5 by volume; 10 ml)) and eluted with hexane + chloroform + acetone (75 + 20 + 5 by volume; 25 ml) at a 10 ml min^{-1} flow rate. The eluant was collected and evaporated to dryness with a rotary vacuum evaporator at 35°C. The residue was dissolved

in methanol (5 ml), filtered through a membrane filter (0·2 μm) and analyzed by liquid chromatography.

2.2.4 Liquid chromatography

The cleaned-up extract was chromatographed using gradient elution: water + acetonitrile + methanol (30 + 30 + 40 by volume, wavelength 245 nm, 0–3 min) to water + acetonitrile + methanol (30 + 30 + 40 by volume, wavelength 230 nm, 3–10 min) to water + acetonitrile + methanol (20 + 20 + 60 by volume, wavelength 230 nm, 10–20 min) at a 1 ml min^{-1} flow rate. Standard solution and analytical sample (10 μl) were injected into the liquid chromatograph. For each sample, peak area responses of fenitrothion (1·55), azinphos-ethyl (1·75), chlorfenson (2·81), chlorobenzilate (3·43), tetradifon (4·79) and dicofol (7·67) were measured at retention times relative to pirimicarb (2·40 min). Using measured responses, linear regression plots of standard curves to determine concentration of pesticides in experimental samples were made. The calibration graphs ($n = 8$) were linear between 0·1 and 10 μg ml^{-1} for pirimicarb and azinphos-ethyl, 0·5 and 20 μg ml^{-1} for fenitrothion and tetradifon, 0·3 and 20 μg ml^{-1} for chlorfenson, 0·5 and 15 μg ml^{-1} for chlorobenzilate and 0·6 and 20 μg ml^{-1} for dicofol.

2.2.5 Gas chromatography

Standard solutions and analytical samples (1 μl) were injected into the gas chromatograph fitted with BP5 capillary column. For each sample, peak area responses of fenitrothion (1·30), azinphos-ethyl (4·86), chlorfenson (2·15), chlorobenzilate (2·65), tetradifon (4·42) and dicofol (1·49) were measured at retention times relative to pirimicarb (4·39 min). Using measured responses, linear regression plots of standard curves to determine concentration of pesticides in experimental samples were made. The calibration graphs ($n = 8$) were linear between 2 and 10 μg ml^{-1} for pirimicarb, 0·5 and 10 μg ml^{-1} for fenitrothion and dicofol, 0·1 and 10 μg ml^{-1} for chlorfenson and tetradifon, 0·5 and 15 μg ml^{-1} for chlorobenzilate and 5 and 15 μg ml^{-1} for azinphos-ethyl.

3 RESULTS AND DISCUSSION

3.1 Liquid chromatography

UV-visible detection is the most commonly used detector employed for residue analysis by LC. To optimize detection, the absorption spectra of the pesticides were obtained. The maximum absorption wavelength of each pesticide was optimized representing the absorption spectra as a contour plot. Figure 1 shows the contour plot of several spectra collected during chromatographic elution using a time interval in the events program of the diode-array detector of 20 spectra per minute. From this, a wavelength program providing optimum resolution of each pesticide component can be deduced. Table 1 lists the wavelengths selected.

Optimization of chromatographic parameters was performed by searching for the separation of the peaks corresponding to each pesticide, allowing a separate peak integration. In Table 2, the retention time t_R, capacity factor k and separation factor α, achieved under two different conditions, A and B, are indicated. In conditions A, a gradient elution at a constant flow

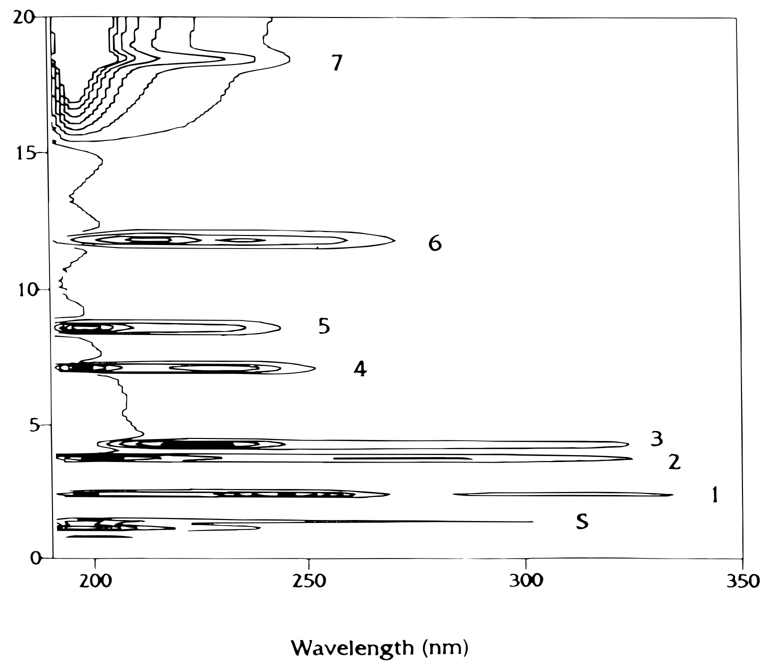

Fig. 1. Contour plot of the chromatographic elution of (1) pirimicarb, (2) fenitrothion, (3) azinphos-ethyl, (4) chlorfenson, (5) chlorobenzilate, (6) tetradifon and (7) dicofol at 5 μg ml^{-1} each. Conditions: 10 μl injected, flow rate 1 ml min^{-1}.

TABLE 1
Liquid Chromatography Conditions

Conditions	*Time (min.)*	*Wavelength (nm)*	*Flow rate (ml min^{-1})*	*Mobile phase composition (% by volume)* *Water*	*Acetonitrile*	*Methanol*
Gradient elution: A	0·0	245	1·0	30	30	40
	3·0	230	1·0	30	30	40
	10·0	230	1·0	30	30	40
	20·0	230	1·0	20	20	60
Variable flow: B	0·0	245	1·0	30	30	40
	3·0	230	1·0	30	30	40
	5·0	230	1·2	30	30	40
	10·0	230	1·4	30	30	40
	20·0	230	1·2	30	30	40
	25·0	245	1·0	30	30	40

rate was used. In conditions B, a variable flow rate with a constant mobile phase composition was used. The wavelength program used in conditions A and B is described in Table 1. In each case a good separation is achieved. We chose conditions A for the chromatography of pesticides.

3.2 Recovery assays

In the absence of sample matrix, the effect of all the pesticides on the quantitation of each other was evaluated. Synthetic mixtures were prepared using a fixed quantity of the pesticide to be recovered, namely pirimicarb 5, azinphos-ethyl 5, fenitrothion 10, chlorfenson 10, chlorobenzilate 10, tetradifon 10 and dicofol 20 ng, and adding the other six at levels between 10 and 200 ng (Table 3). In general, recoveries were consistently good, giving mean values around 100% at the levels assayed for pirimicarb and azinphos-ethyl. Only tetradifon gave mean values of 85% for the 150 ng level. Dicofol, fenitrothion, chlorfenson and chlorobenzilate gave mean values slightly high for the studied levels.

Samples of high fat content are of particular concern because of their propensity to accumulate many of these contaminants. The first step applied to such samples is the extraction of the lipid material from the homogenized sample matrix. Isolation of the lipid-soluble contaminants from the fatty extract for subsequent determination is the second step.

The clean-up step can be carried out in different ways.[16] Gel permeation columns commonly used for separating high-molecular-weight polymers, have been adapted to lipid sample clean-up and are applied to the clean-up of pesticides in fats and oils of both plant and animal origin.[17] This clean-up system was evaluated for the pesticides studied in this work. After the gel permeation column step, further clean-up was advisable for multi-residue analysis to remove interference and improve sensitivity. We selected silica Sep-Pak cartridges because the animal tissue extracts, after gel permeation column, were in a polar solvent.

In Fig. 2, the chromatograms of a liver and a brain extract (spiked and control) are shown. Percentage recoveries were determined in three different liver and brain samples which were each spiked prior to extraction at three levels, 0·25, 1·00 and 2·50 mg kg^{-1}, after checking the sample mix for the absence of the pesticides under study. After extraction the samples were cleaned-up by the gel-permeation-column Sep-Pak pro-

TABLE 2
Effect of Different Conditions in Separation Achieved by Liquid Chromatography

Compounds	*Gradient elution: A* t_R*(min)*	k	α	*Variable flow: B* t_R*(min)*	k	α
Pirimicarb	2·40	1·32		2·24	1·24	
Fenitrothion	3·72	2·81	2·13	3·62	2·63	2·12
Azinphos-ethyl	4·20	3·22	1·15	4·08	3·08	1·17
Chlorfenson	6·75	6·09	1·89	6·32	5·32	1·73
Chlorobenzilate	8·24	7·57	1·24	7·46	6·46	1·21
Tetradifon	11·50	11·10	1·46	9·81	8·81	1·36
Dicofol	18·40	17·12	1·54	15·07	14·07	1·60

TABLE 3
Interference Study

Amount of each other pesticide (ng)	*Recovery of the pesticides in synthetic mixtures (%)*						
	Pirimicarb	*Azinphosethyl*	*Fenitrothion*	*Chlorfenson*	*Chlorobenzilate*	*Tetradifon*	*Dicofol*
10	100	100	100	100	100	100	101
50	100	98	103	103	104	98	105
100	104	102	105	101	107	92	104
150	96	104	106	108	109	85	104
200	94	92	105	107	108	85	104

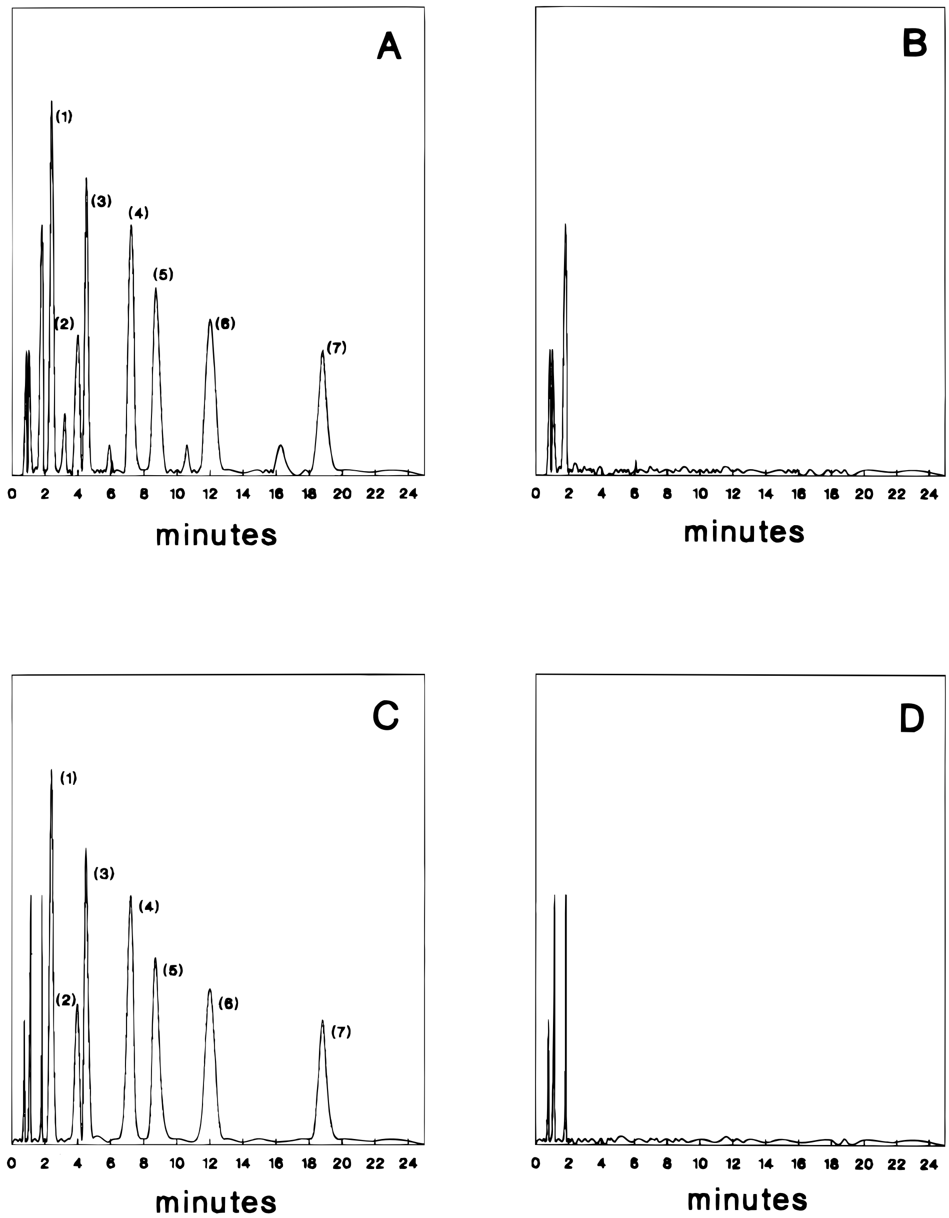

Fig. 2. Liquid chromatographic separation of (1) pirimicarb, (2) fenitrothion, (3) azinphos-ethyl, (4) chlorfenson, (5) chlorobenzilate, (6) tetradifon and (7) dicofol (1 mg kg^{-1} each) of (A) liver and (C) brain extracts. (B) control liver chromatogram and (D) control brain chromatogram.

TABLE 4
Recoveries of Pesticides by Liquid Chromatography from Spiked Liver and Brain Samples

		Recovery (%)							
		Liver				*Brain*			
Compounds	*Added* (*mg kg^{-1}*)	*Sample 1*	*Sample 2*	*Sample 3*	*Mean*	*Sample 1*	*Sample 2*	*Sample 3*	*Mean*
Pirimicarb	0·25	114·0	95·3	98·2	102·5	99·6	93·5	96·7	96·6
	1·0	94·5	97·1	93·2	94·9	99·7	95·0	97·5	97·4
	2·5	99·5	100·6	98·0	99·4	99·7	90·8	95·1	95·2
Fenitrothion	0·25	89·3	103·5	92·0	100·3	97·0	95·0	100·2	97·4
	1·0	99·5	97·9	99·2	98·1	98·4	97·0	95·8	97·1
	2·5	98·4	107·7	101·3	102·8	99·6	96·7	99·7	98·7
Azinphos-ethyl	0·25	102·7	100·5	97·7	94·9	99·8	95·0	93·1	96·0
	1·0	95·1	99·4	99·7	98·8	99·3	95·0	98·8	97·7
	2·5	98·4	105·4	104·6	102·5	98·9	98·4	97·3	96·0
Chlorfenson	0·25	96·0	95·4	106·2	99·2	99·2	98·4	98·8	98·8
	1·0	92·5	108·7	108·5	103·2	97·0	95·0	96·0	96·0
	2·5	95·9	96·0	101·5	97·8	96·3	101·9	98·5	98·9
Chlorobenzilate	0·25	106·7	97·0	113·9	105·8	98·7	101·6	100·0	100·1
	1·0	108·7	111·0	97·6	105·8	98·1	106·0	102·2	102·1
	2·5	97·0	93·2	97·3	95·8	99·9	95·4	97·5	97·6
Tetradifon	0·25	103·3	92·2	93·3	96·3	98·2	93·7	95·8	95·9
	1·0	98·7	98·1	100·0	98·9	94·7	92·5	93·6	93·6
	2·5	98·8	96·9	97·4	97·7	98·9	96·7	97·8	97·8
Dicofol	0·25	106·7	103·0	99·0	102·9	96·1	95·0	95·6	95·6
	1·0	97·7	104·4	107·2	103·1	95·0	105·0	100·0	100·0
	2·5	97·2	99·8	100·5	99·2	99·3	94·5	96·9	96·9

cedure and the results are shown in Table 4. The recoveries obtained from spiked brain and liver samples are excellent, giving mean values around 100% with recoveries ranging from 89·3 to 114·0%.

Since GC is the most commonly used technique in pesticide residue analysis, the results obtained by the LC method were compared to those from an optimized GC method. Although the selective detectors used in gas chromatography permit very sensitive determinations, the methodology for the analysis of different classes of pesticides is laborious. On the other hand, an electron-capture detector (ECD) permits easy measuring of the seven compounds with an adequate sensitivity. In Fig. 3, the gas chromatograms of a liver and brain extract are shown. By using the ECD and the described extraction procedure, recoveries of pesticides in brain and liver samples were consistently good giving mean values around 100% with recoveries ranging from 87·9 to 107·0% (Table 5). Low levels of pirimicarb and azinphos-ethyl were not detected, because the detection limit was greater than 0·25 mg kg^{-1} for pirimicarb and 1 mg kg^{-1} for azinphos-ethyl.

In Table 6, the detection limit values for all the pesticides are shown. In the LC method the detection limits ranged from 0·10 mg kg^{-1} for tetradifon to 0·23 mg kg^{-1} for chlorobenzilate in spiked liver samples and 0·05 mg kg^{-1} for chlorfenson to 0·23 mg kg^{-1} for chlorobenzilate in spiked brain samples. In the GC method the detection limits ranged from 0·05 mg kg^{-1} for chlorfenson to 2·03 mg kg^{-1} for azinphos-ethyl in spiked liver samples and 0·04 mg kg^{-1} for tetradifon to 1·93 mg kg^{-1} for azinphos-ethyl in spiked brain samples. These results indicate that the detection limits of pesticides for the liquid and gas chromatographic methods were comparable, although the LC method was more sensitive than the GC method for pirimicarb and azinphos-ethyl.

CONCLUSIONS

Liquid chromatography is a useful technique for the quantitation of pesticide residues in animal tissues. The results compare well with those obtained by a gas chromatographic method. Recoveries of pesticides from spiked liver and brain samples are consistently good in both methods. So, the HPLC method is an adequate

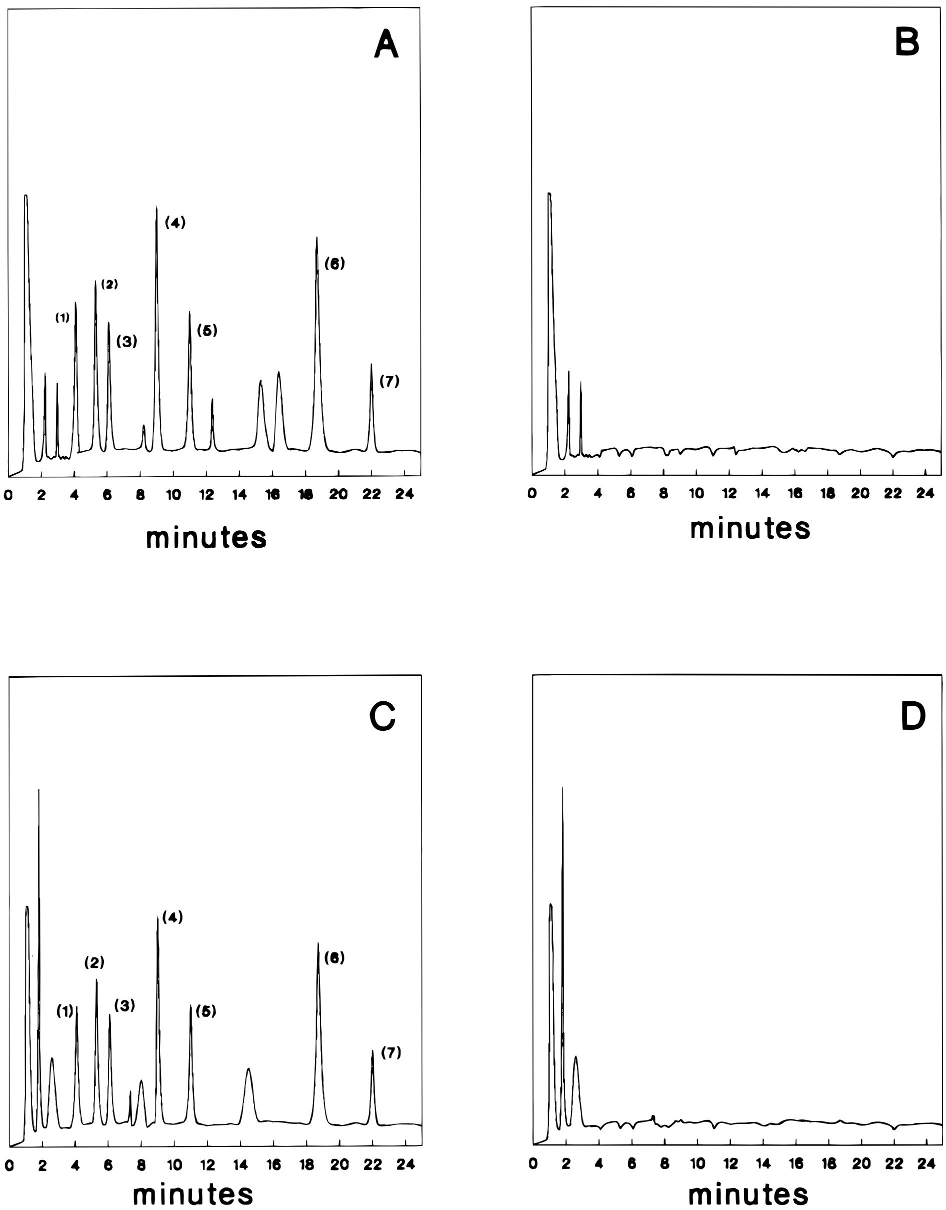

Fig. 3. Gas chromatographic separation of (1) pirimicarb (1 mg kg^{-1}), (2) fenitrothion (1 mg kg^{-1}), (3) dicofol (1 mg kg^{-1}), (4) chlorfenson (1 mg kg^{-1}), (5) chlorobenzilate (1 mg kg^{-1}), (6) tetradifon (1 mg kg^{-1}) and (7) azinphos-ethyl (2·5 mg kg^{-1}) of (A) liver and (C) brain extracts. (B) control liver chromatogram and (D) control brain chromatogram.

alternative to GC for the analysis of different classes of pesticide residues.

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TABLE 5
Recoveries of Pesticides by Gas Chromatography from Spiked Liver and Brain Samples

Compounds	Added ($mg\ kg^{-1}$)	*Recovery* (%) *Liver* Sample 1	Sample 2	Sample 3	Mean	*Brain* Sample 1	Sample 2	Sample 3	Mean
Pirimicarb	1·0	101·5	107·0	99·5	95·2	91·2	99·5	95·0	102·6
	2·5	98·0	92·3	94·7	93·6	94·2	96·7	89·7	95·0
Fenitrothion	0·25	101·8	95·6	87·9	98·6	99·0	101·5	95·3	96·1
	1·0	95·7	88·5	88·7	101·2	99·5	106·5	97·7	91·0
	2·5	96·0	96·6	102·1	99·1	99·4	105·5	92·2	98·2
Azinphos-ethyl	2·5	98·62	97·8	99·0	97·6	98·4	97·8	96·6	98·5
Chlorfenson	0·25	101·18	97·2	94·5	94·6	92·9	95·8	95·1	97·6
	1·0	99·93	99·9	98·8	97·4	98·8	98·2	95·0	99·5
	2·5	100·87	98·5	98·7	95·6	93·0	101·8	92·2	99·4
Chlorobenzilate	0·25	98·1	100·0	99·1	97·4	99·5	96·3	96·2	99·1
	1·0	98·0	100·4	99·1	97·2	99·2	97·5	95·0	99·2
	2·5	95·2	100·4	99·9	94·9	96·6	94·7	93·5	98·5
Tetradifon	0·25	105·0	95·9	99·7	96·4	98·9	96·2	95·0	100·2
	1·0	94·0	102·4	95·9	99·2	100·3	99·2	98·15	97·4
	2·5	91·7	98·5	98·6	95·6	96·2	97·0	93·5	96·2
Dicofol	0·25	95·2	88·3	86·2	96·3	94·4	100·1	94·4	89·9
	1·0	97·0	90·5	95·3	100·5	100·0	103·4	98·0	94·3
	2·5	98·3	98·9	98·5	95·0	97·3	98·3	89·4	98·6

TABLE 6
Detection Limits of Pesticides in Liver and Brain Extracts by Liquid and Gas Chromatography

Compounds	*Liquid chromatography* Liver extract $D_L{}^a$ ($mg\ kg^{-1}$)	Brain extract D_L ($mg\ kg^{-1}$)	*Gas chromatography* Liver extract D_L ($mg\ kg^{-1}$)	Brain extract D_L ($mg\ kg^{-1}$)
Pirimicarb	0·17	0·17	0·46	0·53
Fenitrothion	0·15	0·18	0·17	0·17
Azinphos-ethyl	0·14	0·11	2·03	1·93
Chlorfenson	0·16	0·05	0·05	0·08
Chlorobenzilate	0·23	0·23	0·23	0·26
Tetradifon	0·10	0·08	0·11	0·04
Dicofol	0·18	0·20	0·19	0·25

[a] D_L (Detection limit) defined as the amount that gave a signal-to-noise ratio of three.

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